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13. ABSTRACT (Maximum 200 Words) Nearly 100% of men who die of prostate cancer suffer from painful bone metastases. A major question in prostate cancer biology is why prostate cancer preferentially metastasizes to bone. A better understanding of how PC metastasizes to bone is the first step in defining a therapeutic strategy to block the development of these metastases. In this last period, we have: Task 1: Investigated how the bone microenvironment modulates growth and adhesion of VCaP versus DuCaP by investigating the effects of known bone marrow cytokines and bone ECM on the growth of VCaP and DuCaP. Task 2: We have begun to investigate the differential gene expression of VCaP versus DuCaP cells by cDNA microarrays to identify specific gene products important in the metastasis of prostate cancer to bone by gene profiling.				
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Introduction

Understanding the biology of prostate cancer as well as defining new therapeutic strategies for systemic disease are national priorities in battling this cancer which kills approximately 40,000 men annually. No chemotherapeutic or systemic radiologic modality has been demonstrated to increase survival in prostate cancer patients with metastatic disease. New paradigms of treatment need to be defined. Prostate cancer metastasis to bone represents a unique biological phenomenon that leads to tremendous morbidity and mortality. Nearly 100% of men who die of prostate cancer suffer from metastases to the bone and nearly all of them have required narcotics to treat the pain associated with these lesions. **We hypothesized that there is a difference in the biology of prostate cancer cells that metastasize to bone versus soft tissue sites.** Over the last year, we have utilized bone-derived (VCaP) and dura-derived (DuCaP) prostate cancer cell lines as tools to investigate this hypothesis. DuCaP and VCaP cells are derived from the same patient, meaning they started out fundamentally genetically similar. These two cell lines, however, metastasized to different areas of the body. VCaP went to a common site (the spine) and DuCaP went to an uncommon site (the dura). These two cell lines represent a unique system to study the phenomenon of prostate cancer cell metastasis to bone by studying what is similar and different between them.

Body

OBJECTIVES: To identify factors involved in organ-specific metastasis, we used cDNA microarray analysis to compare VCaP and DuCaP. Forty-five genes were differentially expressed, and only seven of these also had increased expression in VCaP compared with normal prostatic tissue. Of these, protease-activated receptor 1 (PAR1) was verified as having increased expression by reverse transcriptase-polymerase chain reaction and Northern blot analysis, as well as by immunohistochemistry. PAR1 expression in a panel of prostate cancer cell lines demonstrated increased expression in those cell lines derived from bone metastases. Alpha-thrombin stimulation of the VCaP cells produced a dose-dependent mobilization of intracellular calcium compared with DuCaP, suggesting that PAR1 expressed on the VCaP prostate cancer cell line is functional. These data indicate that a functional PAR1 is expressed on prostate cancer cell lines. The prostate cancer cell lines expressing PAR1 appear to have an association with increased bone metastases.

Prostate cancer metastasis to bone may be mediated by preferential proliferation of these cells in the bone's microenvironment. We hypothesize that this preferential proliferation is mediated by bone-associated growth factors (GFs) and cytokines. To test our hypothesis, human prostate cancer cells, derived from both soft tissue (LNCaP, DuCaP, DU145) and bone metastases (PC-3, VCaP, MDA-2a, MDA-2b), were treated with bone-associated GFs and cytokines (PDGF, IGF-1, TGF-beta, EGF, bFGF, TNF-alpha, IL-1, and IL-6) for 48 h, and their growth responses were compared. The responses of soft tissue-derived prostate cancer cell lines to bone GFs and cytokines were variable. LNCaP cell growth was stimulated by IGF-1 but was inhibited by TNF-alpha. DU145 cell growth was stimulated with EGF. Prostate cancer cell lines derived from bone metastases also responded variably to bone GFs and cytokines. IL-1 stimulated the growth of MDA-2a and 2b cell lines in a dose-dependent manner. PDGF and bFGF both demonstrated variable effects on bone-derived prostate cancer cell lines. TNF-alpha inhibited proliferation of the VCaP cells. These findings demonstrate that human prostate cancer cell lines derived from bone metastases may not respond preferentially to bone-associated GFs and cytokines.

Key Research accomplishments

- VCaP overexpresses PAR-1
- Human prostate cancer cell lines derived from bone metastases may not preferentially respond to bone associated growth factors and cytokines.

Reportable outcomes

Personnel

This award provides salary support for Chris Neeley and Martha Davis-Merritts who assist Dr. Pienta in these studies.

Manuscripts

Chay CH, Cooper CR, Gendernalik JD, Dhanasekaran SM, **Chinnaiyan AM**, Rubin MA, Schmaier AH, **Pienta KJ**. A functional thrombin receptor (PAR1) is expressed on bone-derived prostate cancer cell lines. *Urology* 60:760-765, 2002.

Lee HL, **Pienta KJ**, Kim WJ, Cooper CR. The effect of bone associated growth factors and cytokines on the growth of prostate cancer cells derived from soft tissue versus bone metastases in vitro.

Conclusions

Although early in the award process, we have already made key discoveries related to prostate cancer cell-bone microenvironment interactions. We will continue to investigate the biologic relevance of PAR1 expression in VcaP versus DuCaP cells. We will investigate differences in adhesion to HBME cells between the two cell lines.

References

None

A FUNCTIONAL THROMBIN RECEPTOR (PAR1) IS EXPRESSED ON BONE-DERIVED PROSTATE CANCER CELL LINES

CHRISTOPHER H. CHAY, CARLTON R. COOPER, JAMES D. GENDERALIK,
SARAVANA M. DHANASEKARAN, ARUL M. CHINNAIYAN, MARK A. RUBIN, ALVIN H. SCHMAIER,
AND KENNETH J. PIENTA

ABSTRACT

Objectives. To identify genes important in prostate cancer metastatic to bone. Bone-specific metastasis is a common feature of prostate cancer and a significant cause of morbidity.

Methods. To identify factors involved in organ-specific metastasis, we used cDNA microarray analysis to compare a bone-derived cell line, VCaP, with a soft tissue-derived cell line, DuCaP. Both cell lines were derived from the same patient and spontaneously passaged.

Results. Forty-five genes were differentially expressed, and only seven of these also had increased expression in VCaP compared with normal prostatic tissue. Of these, protease-activated receptor 1 (PAR1) was verified as having increased expression by reverse transcriptase-polymerase chain reaction and Northern blot analysis, as well as by immunohistochemistry. PAR1 expression in a panel of prostate cancer cell lines demonstrated increased expression in those cell lines derived from bone metastases. Alpha-thrombin stimulation of the VCaP cells produced a dose-dependent mobilization of intracellular calcium compared with DuCaP, suggesting that PAR1 expressed on the VCaP prostate cancer cell line is functional.

Conclusions. These data indicate that a functional PAR1 is expressed on prostate cancer cell lines. The prostate cancer cell lines expressing PAR1 appear to have an association with increased bone metastases. UROLOGY 60: 760-765, 2002. © 2002, Elsevier Science Inc.

Prostate cancer metastasis to bone not only occurs early in the course of disease, but is the most common site of metastasis.^{1,2} Clinical patterns of metastasis have been recognized for many years, but the interactions between prostate cancer

cells and the bone marrow that allow for this specific and frequent finding remain poorly understood. Microarray analysis has recently been used to compare prostate cancer from patients and cell lines with normal prostatic tissue.^{3,4} Although cDNA microarrays can be used to characterize cell lines and patient samples, no studies have addressed the differences in expression patterns important in determining organ-specific metastasis.

It has been increasingly recognized that hemostatic proteins and mechanisms associated with vascular biology are associated with cancer metastasis.⁵ Although cancer cells can be associated with hypercoagulability, evidence is growing that hemostatic factors, including thrombin, may increase metastasis through adhesion and invasion. The thrombin receptor or protease-activated receptor 1 (PAR1) has been identified on platelets, endothelial cells, smooth muscle cells, and fibroblasts. Recent studies have identified functional thrombin receptors on cancer cells as well. Thrombin signal-

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A. H. Schmaier holds stock in, and is President of, Thromgen, Inc.

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TABLE 1. cDNA microarray analysis comparing VCaP and DuCaP gene expression

Gene Name	VCaP vs. DuCaP	VCaP vs. Control
Folate hydrolase (prostate-specific membrane antigen) 1	3.046	1.035
Nectin 3; DKFZP566B0846 protein	3.03	1.16
EST H75860*	2.634	1.99
Hypothetical protein FLJ20510	2.382	1.414
p75NTR-associated cell death executor; ovarian granulosa cell protein (13kD)	2.37	1.15
Six transmembrane epithelial antigen of the prostate*	2.369	1.673
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)*	2.289	1.893
KIAA1200 protein*	2.27	1.88
FAT tumor suppressor (Drosophila) homolog	2.254	0.433
Uncharacterized hypothalamus protein HBEX2*	2.17	2.05
Coagulation factor II (thrombin) receptor*	2.168	2.259
Hypothetical protein FLJ20624	2.162	
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 17 (72 kDa)	2.131	0.856
<i>Homo sapiens</i> mRNA for KIAA1727 protein, partial cds	2.123	0.908
Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	2.123	0.542
<i>Homo sapiens</i> cDNA FLJ13555 fis, clone PLACE1007677	2.043	1.147
Guanylate cyclase 1, soluble, alpha 3*	2.043	1.681
Inositol polyphosphate phosphatase-like 1	2.043	0.542
Frizzled (Drosophila) homolog 4	2.032	1.063
GS3955 protein	2.024	1.444
Hypothetical protein	2.023	1.252
EST N93721	2.022	0.711
Purkinje cell protein 4	2.017	0.995
	VCaP vs. DuCaP	DuCaP vs. Control
Ribosomal protein S14	0.498	1.422
ESTs, weakly similar to T17346 hypothetical protein DKFZp586O1624.1 [<i>H. sapiens</i>]	0.496	1.443
Sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	0.494	0.604
KIAA0758 protein	0.493	0.657
Fibronectin 1	0.489	0.182
Melanoma adhesion molecule	0.489	0.171
ESTs, weakly similar to ALUB_HUMAN !!!! ALU CLASS B WARNING ENTRY !!! [<i>H. sapiens</i>]	0.489	1.394
Neuronal cell adhesion molecule*	0.475	3.014
ESTs, weakly similar to JC1087 RNA helicase, ATP-dependent [<i>H. sapiens</i>]	0.474	1.131
Transmembrane 4 superfamily member 1*	0.468	2.429
Killer cell lectin-like receptor subfamily C, member 2	0.453	0.91
Mesenchymal stem cell protein DSC54*	0.453	1.676
Hypothetical protein FLJ11238*	0.443	2.701
<i>Homo sapiens</i> sprouty-4C mRNA, complete cds	0.441	1.068
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	0.437	0.497
KIAA0275 gene product*	0.434	1.738
Nucleosome assembly protein 1-like 1*	0.433	4.004
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	0.432	0.764
Hexabrachion (tenascin C, cytactin)	0.418	0.42
T-cell receptor gamma locus*	0.385	3.776
EST R93401	0.363	1.406
Vimentin	0.259	0.595

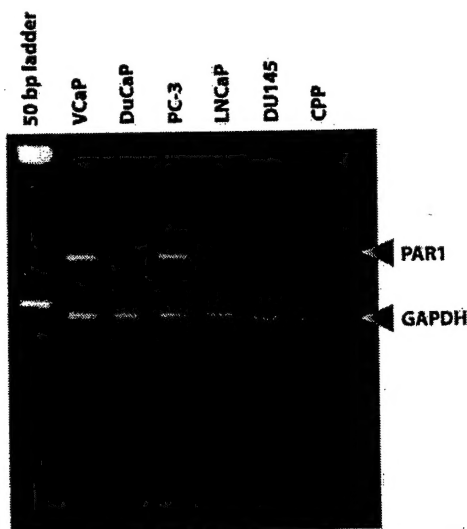
Comparing the expression between the VCaP and DuCaP cell lines, 45 genes were identified as being differentially expressed using a twofold cutoff. Although 23 genes had increased expression in VCaP compared with DuCaP, only 7 of these genes also had increased expression compared with a normal prostate control sample using a 1.5-fold cutoff (denoted by asterisk).

ing through PAR1 has been shown to increase adhesion to fibronectin,⁶ invasiveness,⁷ and the development of murine pulmonary metastases.⁸

In an attempt to identify genes that may be involved in prostate cancer metastasis to bone, we

used cDNA microarray analysis to compare the gene expression of VCaP, a bone-derived prostate cancer cell line,⁹ and DuCaP, a soft tissue-derived prostate cancer cell line.¹⁰ The VCaP and DuCaP cell lines were derived from the same patient and

A.



B.

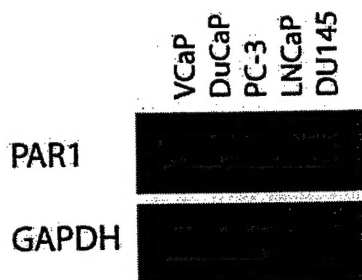


FIGURE 1. (A) Semiquantitative multiplex RT-PCR of PAR1 revealed increased expression in VCaP compared with DuCaP by 2.5-fold. PC-3 cells also had a twofold increase in expression compared with LNCaP and a sixfold increase compared with DU145. (B) Northern blot analysis revealed a similar pattern in PAR1 expression as RT-PCR with increased expression in the VCaP and PC-3 cell lines compared with the DuCaP, LNCaP, and DU145 cell lines.

both were spontaneously passaged. This report verifies that PAR1 is upregulated in VCaP compared with DuCaP and demonstrates that PAR1 has increased expression in PC-3, another bone-derived prostate cancer cell line.

MATERIAL AND METHODS

TUMOR CELL LINES

The PC-3, LNCaP, and DU145 cell lines were obtained from ATCC (Rockville, Md) and maintained in RPMI medium. The VCaP and DuCaP cell lines were maintained in Dulbecco's modified Eagles medium and grown as previously described.^{9,10} The normal human lung fibroblast (NHLF) cell

line (Clonetics, Walkersville, Md) was maintained in fibroblast growth media-2 (FGM-2) media.

CDNA MICROARRAY

A 9978 element human cDNA microarray from Research Genetics (Huntsville, Ala) human cDNA clone set was studied using RNA from VCaP and DuCaP, as previously described.³ A reference sample of commercially available normal prostatic tissue (Clonotech, Palo Alto, Calif) used in these experiments was labeled using a distinguishable fluorescent dye.

SEMIQUANTITATIVE MULTIPLEX POLYMERASE CHAIN REACTION

RNA was prepared by the standard TriZol protocol (Life Technologies, Rockville, Md) using each of the cell lines. Equal amounts of RNA were used to produce cDNA using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, Calif). For the amplification of PAR1, 200 ng each of the forward primer GTGCTGTTTGTGTCTGTGCT and the reverse primer CCTCTGTGGTGAAGTGTGA was used in addition to 2 pmol each of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer GTGAAGGTCGGAGTCAACG and reverse primer GGTGAAGACGCCAGTGGACTC.

NORTHERN BLOT ANALYSIS

RNA expression patterns were analyzed using standard Northern blot analysis with a PAR1 probe. The blot was then probed again for GAPDH.

IMMUNOHISTOCHEMISTRY

All cell lines were fixed in ethanol, embedded in paraffin, and subsequently immunohistochemically stained by the avidin-biotin complex technique with a monoclonal antibody for PAR1, Thrombin R (ATAP2, Santa Cruz Biotechnology, Santa Cruz, Calif). The antibody was diluted 1:25, and staining was performed on an automated stainer (Dako, Carpinteria, Calif). The negative control was obtained by omitting the primary antibody.

CALCIUM MOBILIZATION

The cell lines VCaP and DuCaP were washed in HEPES-Tyroses buffer, loaded with 5 μ M fura-2 AM (Molecular Probes, Eugene, Ore), and placed in a thermostatically controlled spectrofluorophotometer (PerkinElmer, LS50 Spectrofluorometer).¹¹ The excitation wavelengths varied between 340 and 380 nm. The fluorescence was measured by recording emitted light at 510 nm. Alpha-thrombin was added in concentrations varying from 1 to 8 nm. The MAP4-RPPGF,¹² a peptide inhibitor of PAR1, was added at concentrations ranging from 3 to 100 μ M.

RESULTS

CDNA MICROARRAY COMPARING VCAP WITH DUCAP

In comparing the expression between the VCaP and DuCaP cell lines, only 45 genes were identified as being differentially expressed using a twofold cutoff in expression (Table I). Twenty-three genes had increased expression in VCaP, and 22 genes had increased expression in DuCaP. The expression of these cell lines was also compared with commercially available normal prostatic tissue using a 1.5-fold cutoff. Of the 45 genes initially identified, only 14 genes met both criteria. Seven genes had increased expression in VCaP compared with

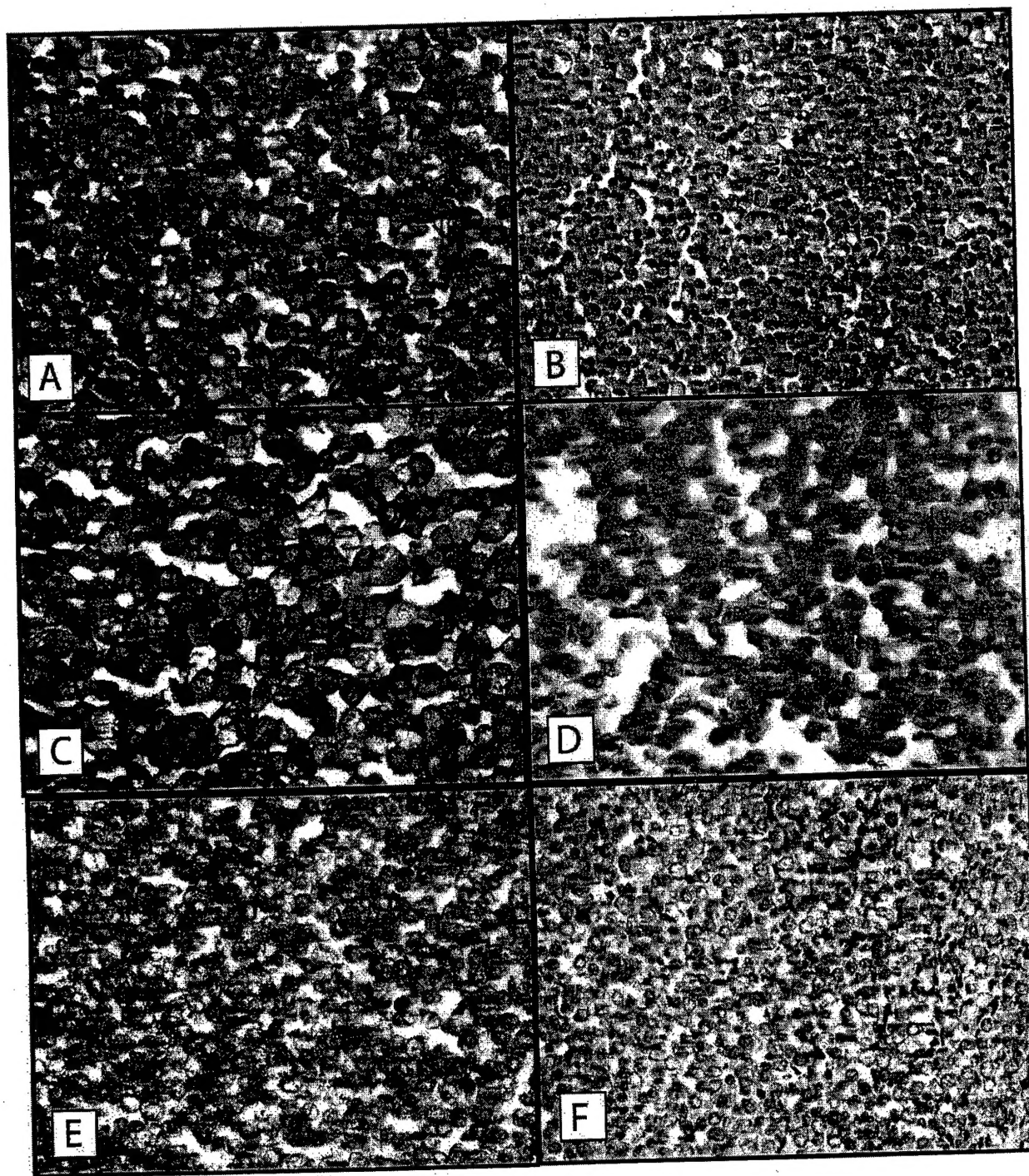


FIGURE 2. Immunohistochemistry using a PAR1-specific antibody demonstrated membrane staining in (A) the positive control NHLF cell line compared with (B) the negative control PC-3 cell line without the primary antibody. The PC-3 and VCaP cell lines had strong membrane staining (C,E) compared with the DU145 and DuCaP cell lines (D,F).

DuCaP, as well as the normal prostate control (denoted by asterisks in Table I). Six transmembrane epithelial antigens of the prostate were identified and have been previously described as expressed in prostate cancer. In addition, the neuronal cell adhesion molecule was identified as having increased expression in DuCaP. PAR1 was identified as having increased expression in VCaP.

VERIFICATION OF PAR1 EXPRESSION

PAR1 was selected for additional study, because it is a transmembrane protein and has been associated with increased invasiveness in other cancer models.⁷ Semiquantitative multiplex RT-PCR was performed using PAR1 and GAPDH primers. PAR1 was found to have increased expression in VCaP compared with DuCaP by approximately 2.5-fold

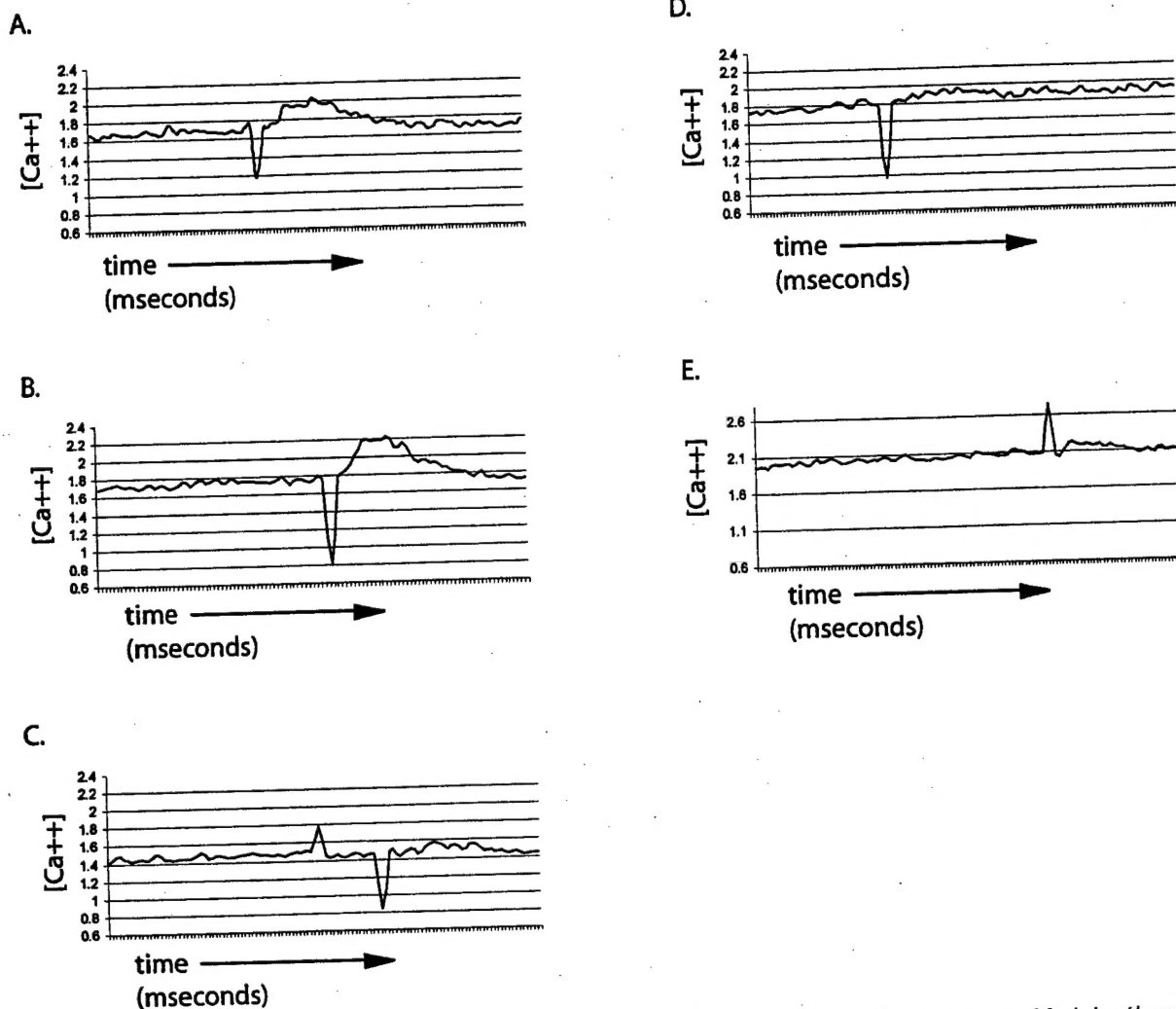


FIGURE 3. Calcium mobilization studies. (A) VCaP plus 2 nM alpha-thrombin, (B) VCaP plus 4 nM alpha-thrombin, (C) VCaP plus 2 nM alpha-thrombin plus 50 μ M MAP4-RPPGF, (D) DuCaP plus 2 nM alpha-thrombin, and (E) DuCaP plus 8 nM alpha-thrombin.

(Fig. 1A). In addition, PAR1 expression in PC-3 was 2.0-fold greater than in LNCaP and 6.0-fold greater than in DU145. The expression pattern by Northern blot analysis was consistent with the findings by RT-PCR (Fig. 1B). This verified that PAR1 has increased expression in VCaP compared with DuCaP and that its expression is greatest in the bone-derived cell lines VCaP and PC-3.

IMMUNOHISTOCHEMISTRY

Protein expression in the cell lines was verified by immunohistochemistry. The fibroblast cell line NHLF was used as a positive control and demonstrated strong membrane staining (Fig. 2A). The PC-3 cell line without the primary antibody was used as a negative control (Fig. 2B). PAR1 expression on the PC-3 cell line was increased and revealed strong membrane staining (Fig. 2C) compared with the DU145 cell line (Fig. 2D). The

VCaP cell line also had strong staining (Fig. 2E) compared with the DuCaP cell line, which had faint cytoplasmic staining (Fig. 2F).

CALCIUM MOBILIZATION

The stimulation of the VCaP cell line with 2 nM alpha-thrombin resulted in the mobilization of intracellular calcium (Fig. 3A). This effect of alpha-thrombin was dose dependent, with a significant increase in calcium transients at 4 nM alpha-thrombin (Fig. 3B). MAP4-RPPGF, a specific PAR1 inhibitor, blocked 2 nM alpha-thrombin-induced calcium mobilization (Fig. 3C). The DuCaP cell line had no response to 2 nM or 8 nM alpha-thrombin (Fig. 3D,E). These data demonstrate that the PAR1 expressed on VCaP is functional and that the mobilization of intracellular calcium in response to alpha-thrombin is specific to PAR1.

COMMENT

The biologic role of thrombin has been well characterized in terms of its ability to stimulate platelets and endothelial cells to induce wound healing and angiogenesis. Thrombin elicits a cellular response by cleaving thrombin receptors or PARs.¹³ Recent studies have identified functional thrombin receptors on cancer cells. Wojtukiewicz *et al.*⁶ identified the thrombin receptor in human colon adenocarcinoma cells, as well as murine sarcoma and melanoma cell lines, by RT-PCR and Western blot analysis. Stimulating the cell lines with agonist peptides resulted in dose-dependent increases in adhesion to fibronectin. Fisher and colleagues¹⁴ also identified a thrombin-mediated response with increased thymidine incorporation and calcium mobilization in a human melanoma cell line, M24met. More recently, two groups have associated PAR1 expression with breast cancer invasiveness.^{7,15} Even-Ram and colleagues⁷ demonstrated that thrombin-mediated breast cancer invasion through a biomatrix could be inhibited by PAR1 antisense. Not only has stimulation of PAR1 been shown to be functional on cancer cells, but thrombin has also been shown to increase the *in vivo* development of murine pulmonary metastases. Nierodzik and colleagues⁸ stimulated the B16a melanoma cell line with thrombin and performed tail vein injections in C57Bl/6J mice. They found a sixfold increase in the number of pulmonary metastases and a 68-fold increase in the pulmonary tumor mass. Cumulatively, these studies suggest that some cancer cells may express a functional thrombin receptor that increases their invasiveness and ability to metastasize.

The present study verifies that the VCaP and PC-3 cell lines have increased PAR1 expression compared with both DU145 and LNCaP. Although these data reveal a trend toward increased expression of PAR1 in bone-derived prostate cancer cell lines, PC-3 is known to have increased invasive properties. Clearly, more definitive data are needed before an association between PAR1 expression and patterns in prostate cancer metastasis can be established.

CONCLUSIONS

These data demonstrate that PAR1 is differentially expressed on VCaP, a bone-derived prostate cancer cell line, compared with DuCaP, a soft-tissue derived prostate cancer cell line. PAR1 expression in VCaP has been verified by RNA and protein expression and has been shown to be functional. By directly comparing cell lines derived from two metastatic lesions from the same patient, this study

attempted to understand the biology of organ-specific metastasis. In studying other well-established prostate cancer cell lines, PAR1 appears to have increased expression in prostate cancer cell lines derived from bone metastases. It remains unclear whether the differential expression of PAR1 is related to the ability of a metastatic lesion to go to bone versus soft tissue or represents an increased invasive ability. Future studies have been planned to study the difference in PAR1 expression in metastatic versus primary prostate cancer in patient samples.

ACKNOWLEDGMENT. To Mark Warnock for technical assistance.

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The effect of bone-associated growth factors and cytokines on the growth of prostate cancer cells derived from soft tissue versus bone metastases *in vitro*

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Abstract. Prostate cancer metastasis to bone may be mediated by preferential proliferation of these cells in the bone's micro-environment. We hypothesize that this preferential proliferation is mediated by bone-associated growth factors (GFs) and cytokines. To test our hypothesis, human prostate cancer cells, derived from both soft tissue (LNCaP, DuCaP, DU145) and bone metastases (PC-3, VCaP, MDA-2a, MDA-2b), were treated with bone-associated GFs and cytokines (PDGF, IGF-1, TGF- β , EGF, bFGF, TNF- α , IL-1, and IL-6) for 48 h, and their growth responses were compared. The responses of soft tissue-derived prostate cancer cell lines to bone GFs and cytokines were variable. LNCaP cell growth was stimulated by IGF-1 but was inhibited by TNF- α . DU145 cell growth was stimulated with EGF. Prostate cancer cell lines derived from bone metastases also responded variably to bone GFs and cytokines. IL-1 stimulated the growth of MDA-2a and 2b cell lines in a dose-dependent manner. PDGF and bFGF both demonstrated variable effects on bone-derived prostate cancer cell lines. TNF- α inhibited proliferation of the VCaP cells. These findings demonstrate that human prostate cancer cell lines derived from bone metastases may not respond preferentially to bone-associated GFs and cytokines.

Introduction

Prostate adenocarcinoma (PCa) is consistently recognized as the second leading cause of cancer death in North American

men (1). Despite its common occurrence, the biology of prostate cancer and metastasis remains poorly characterized. As a consequence, prostate tumors, after a relatively short period of regression induced by hormone ablation therapy, continue to grow and metastasize to bone and soft tissues. The skeleton is the most common site for prostate cancer metastasis, occurring in more than 80% of advanced prostate cancer patients (2). The proclivity for prostate cancer to metastasize to bone has been explained by a number of theories, including hemodynamic mechanisms, as well as the 'seed and soil' theory (3-5).

It is established that growth factors (GFs) and cytokines involved in cell proliferation and apoptosis can affect the growth of human PCa cell lines (6). In the bone marrow, several GFs and cytokines are produced by osteoblasts and are incorporated into the bone matrix (7). These bone-associated GFs may support and regulate the growth of PCa cells, even in the absence of androgens. The preferential metastasis to bone by PCa cells suggests that factors in the bone may preferentially stimulate PCa cell growth. The current study determines the role of bone-associated GFs and cytokines by comparing their effects on the growth responses of several soft tissue-derived prostate cancer cell lines to the growth responses of several bone-derived prostate cancer cell lines. In total, seven PCa cell lines were studied with eight different GFs and cytokines. We determined that PCa cell lines derived from bone metastases do not respond preferentially to bone-associated GFs and cytokines as compared to PCa cell lines derived from soft tissue metastases. This study suggests that the sensitivity of prostate cancer cells to various GFs and cytokines is heterogenous at various metastatic sites.

Materials and methods

Cell lines and reagents. LNCaP, DU145, and PC-3 human PCa cell lines were purchased from American Type Culture Collection (Rockville, MD). MDA PCa 2a and 2b human PCa cell lines were developed, characterized and kindly provided by Dr Nora Navone (M.D. Anderson Cancer Center, Houston, TX) (8). DuCaP and VCaP were developed in our laboratory and reported (9,10). LNCaP, DU145, and DuCaP were derived from soft tissue metastases, and PC-3, VCaP, MDA PCa 2a and 2b were derived from bone metastases. LNCaP and PC-3 cells were maintained in RPMI-1640 supplemented with

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Abbreviations: PCa, prostate cancer; EGF, epidermal growth factor; IGF, insulin growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin; NF- κ B, nuclear factor- κ B

Key words: prostate cancer, growth factors, cytokines, bone metastasis

10% fetal bovine serum (FBS) and 1% (vol/vol) penicillin-streptomycin (pen-strep). DU145, DuCaP, and VCaP cells were maintained in DMEM with 10% FBS and 1% pen-strep. MDA PCa 2a and 2b cells were maintained in BRFF-HPCI media (Biological Research Faculty and Facility, Inc., Jamsville, MD) supplemented with 20% FBS and 1% pen-strep. The culture medium was changed every 3-4 days.

The GFs and cytokines used in this investigation were obtained from two commercially available sources. Recombinant human epidermal growth factor (EGF), recombinant human basic fibroblast growth factor (bFGF), recombinant human tumor necrosis factor- α (TNF- α), recombinant human insulin growth factor-1 (IGF-1), recombinant human platelet-derived growth factor (PDGF), and recombinant human transforming growth factor- β 1 (TGF- β) were purchased from Gibco (Grand Island, NY). Recombinant human interleukin-6 (IL-6) and recombinant human interleukin-1 β (IL-1 β) were purchased from R&D systems (Minneapolis, MN).

Proliferation assay. All cells were dispersed by trypsin treatment, resuspended in appropriate media (as detailed above) and seeded into 96-well culture plates at various densities (from 5×10^3 cells per well to 1.5×10^4 cells per well) depending on cell type. Twenty-four hours after seeding the cells, attached cells were rinsed with Hank's balanced salt solution, and the media were changed to both normal media (control) and media containing different concentrations of each exogenous GF and cytokine. Forty-eight hours after the addition of each GF and cytokine, 10 μ l of WST-1 reagent (Roche, Mannheim, Germany) were added to each well and incubated for 3 h at 37°C. The relative growth rate of each cell line was determined with a microtiter plate reader set at wavelengths of 450 nm and 650 nm. The sample size was 10 for each treatment concentration, and all values were compared to their respective control values. Relative scores were calculated and each experiment was repeated twice.

Statistical analysis. A one-way ANOVA with post hoc test (Scheffe adjustment) was used to determine the significance of various treatments versus the control values. Statistically significant values were indicated by $p < 0.05$.

Results

Human prostate cancer cell lines derived from soft tissue metastases: LNCaP, DU145, and DuCaP. The growth responses of LNCaP, DU145, and DuCaP cells were determined. IGF-1 stimulated LNCaP cell proliferation in a dose-independent manner (Fig. 1A and B). The proliferation of LNCaP cells was significantly decreased with TNF- α treatment (Fig. 1A and C). The TNF- α effect was dose-dependent beyond a concentration of 10 U/ml. No significant growth responses were observed when LNCaP cells were treated with PDGF, bFGF, EGF, IL-6, TGF- β , and IL-1.

DU145 and DuCaP cells, both derived from dural metastases, demonstrated variable responses to the cytokines. The growth of DU145 cells was significantly increased with treatments using EGF and two lower concentrations of IGF (Fig. 2A and B). No significant growth responses were noted with bFGF, PDGF, TGF- β , TNF- α , IL-6, and IL-1 treatments

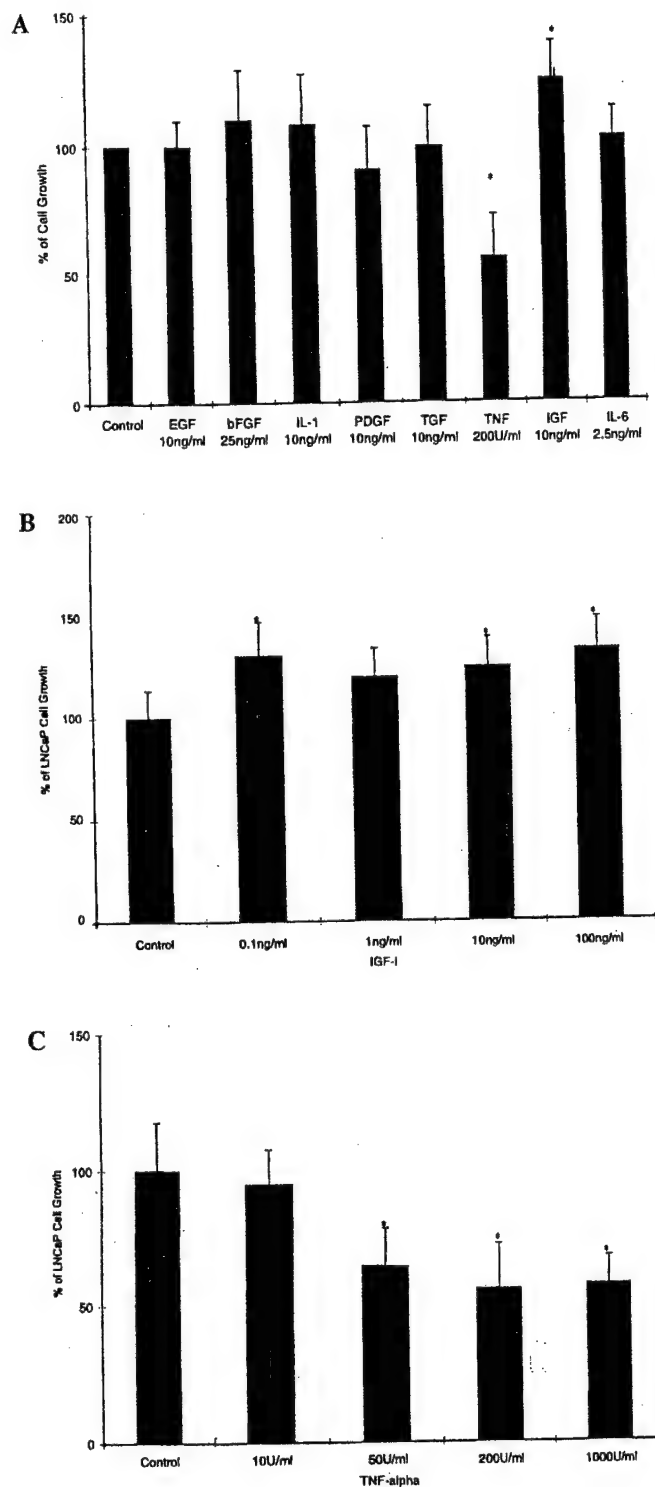


Figure 1. A, The relative growth rate of LNCaP cells incubated for 48 h with each GF/cytokine. B, The growth response of LNCaP cells incubated for 48 h in the presence of 0, 0.1, 1, 10, 100 ng/ml of IGF-1. C, The growth response of LNCaP cells incubated for 48 h in the presence of 0, 10, 50, 200, 1000 U/ml of TNF- α . Results (mean \pm SDM) expressed as the percent of the control, with an asterisk indicating significant values ($p < 0.05$) as compared to the control.

(data not shown). The growth of androgen-sensitive DuCaP cells was only significantly affected by IGF treatment at the highest dose (Fig. 2C).

Human prostate cancer cell lines derived from bone metastases: PC-3, MDA-2a, MDA-2b, and VCaP. The effects

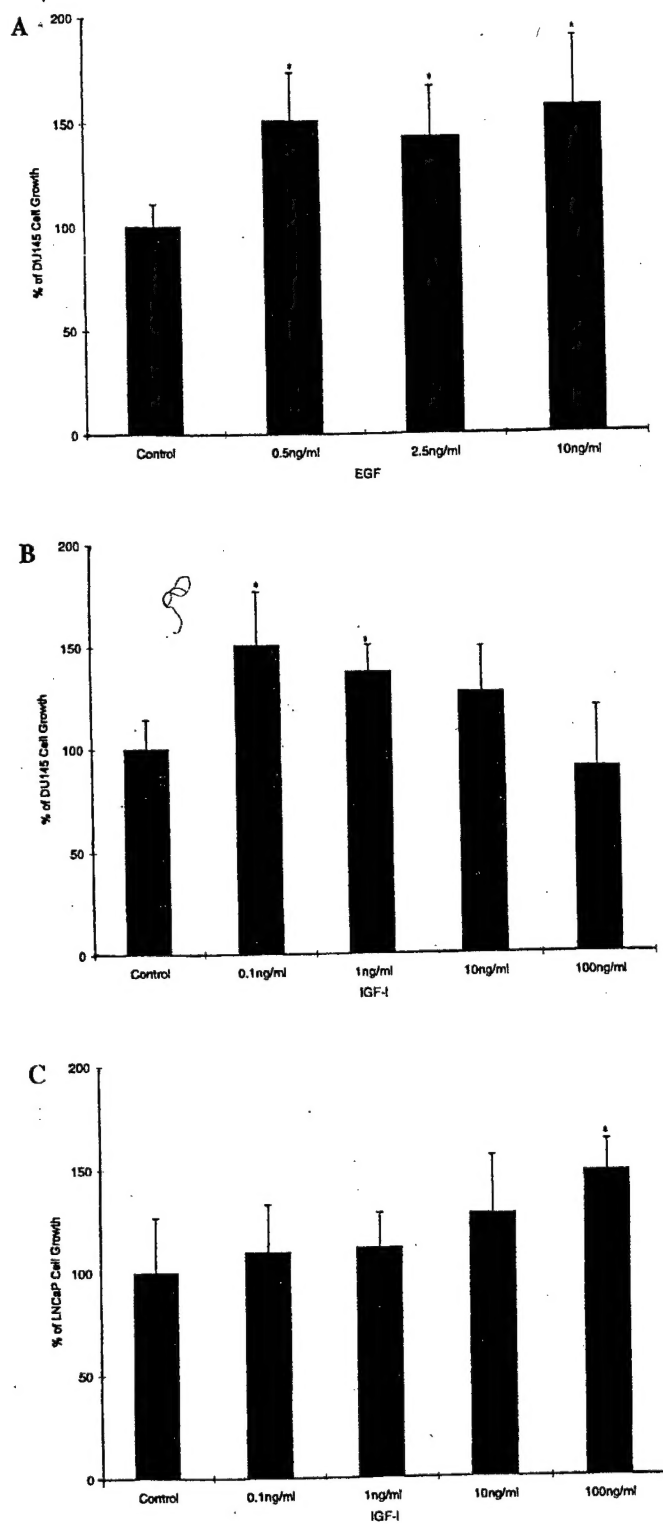


Figure 2. A, The growth response of DU145 cells incubated for 48 h in the presence of 0, 0.5, 2.5, 10 ng/ml of EGF. B, The growth response of DU145 cells incubated for 48 h in the presence of 0, 0.1, 1, 10, 100 ng/ml of IGF-I. C, The growth response of LNCaP cells incubated for 48 h in the presence of 0, 0.1, 1, 10, 100 ng/ml of IGF-I. Results (mean \pm SDM) expressed as the percent of the control, with an asterisk indicating significant values ($p < 0.05$) as compared to the control.

of bone-associated cytokines on bone-derived prostate cancer cell lines, PC-3, VCaP, MDA-2a and MDA-2b, were evaluated. Among the eight cytokines tested, only PDGF had a significant,

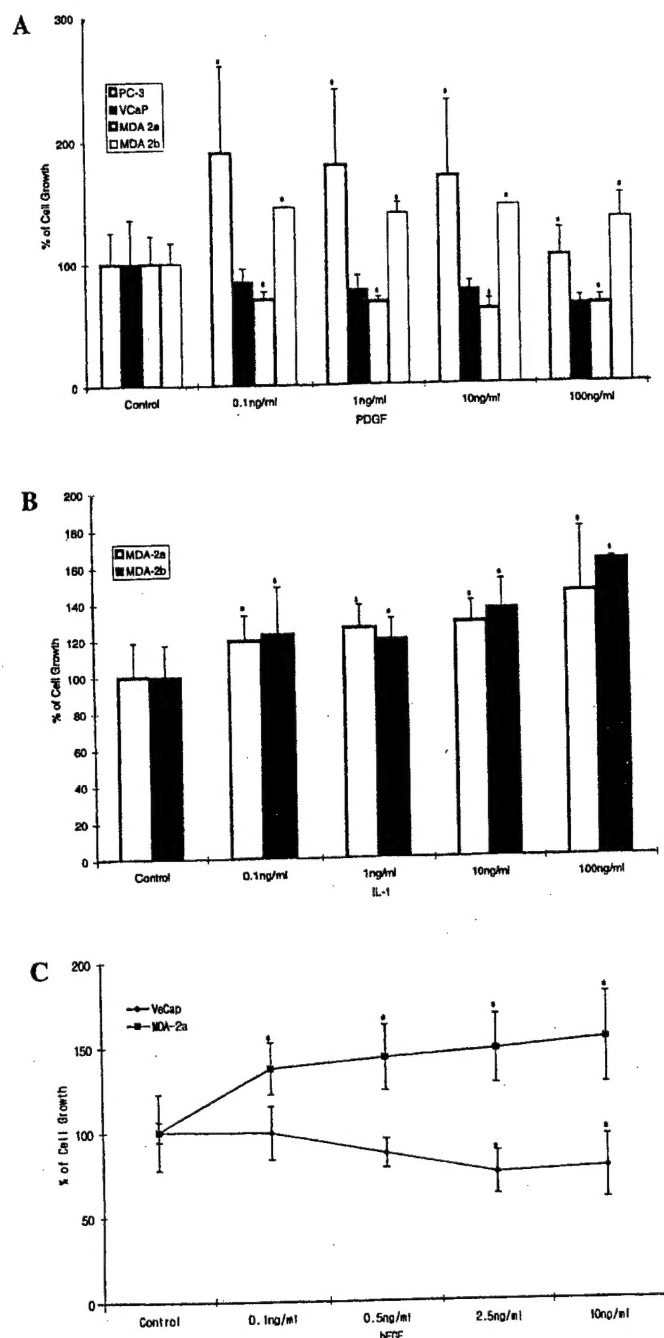


Figure 3. A, The effects of PDGF on the growth of prostate cancer cell lines derived from bone metastases. PC-3 (n=10), VCaP (n=10), MDA-2a (n=10), and 2b (n=10) cells were incubated for 48 h in the presence of 0, 0.1, 1, 10, 100 ng/ml of PDGF. B, The growth responses of MDA-2a and 2b cells incubated for 48 h in the presence of 0, 0.1, 1, 10, 100 ng/ml of IL-1. C, The responses of bFGF on the growth of prostate cancer cells derived from bone metastases. VCaP (n=10) and MDA-2a (n=10) cells were incubated for 48 h in the presence of 0, 0.1, 0.5, 2.5, 10 ng/ml of bFGF. Results (mean \pm SDM) expressed as the percent of the control, with an asterisk indicating significant values ($p < 0.05$) as compared to the control.

but variable, effect on the growth of all cell lines derived from bone metastases (data not shown). Although it was not statistically significant, the growth of VCaP cells was inhibited by PDGF treatment (Fig. 3A). MDA-2a and MDA-2b cells are newly established cell lines and were derived from the same patient (8). Interestingly, PDGF stimulated the

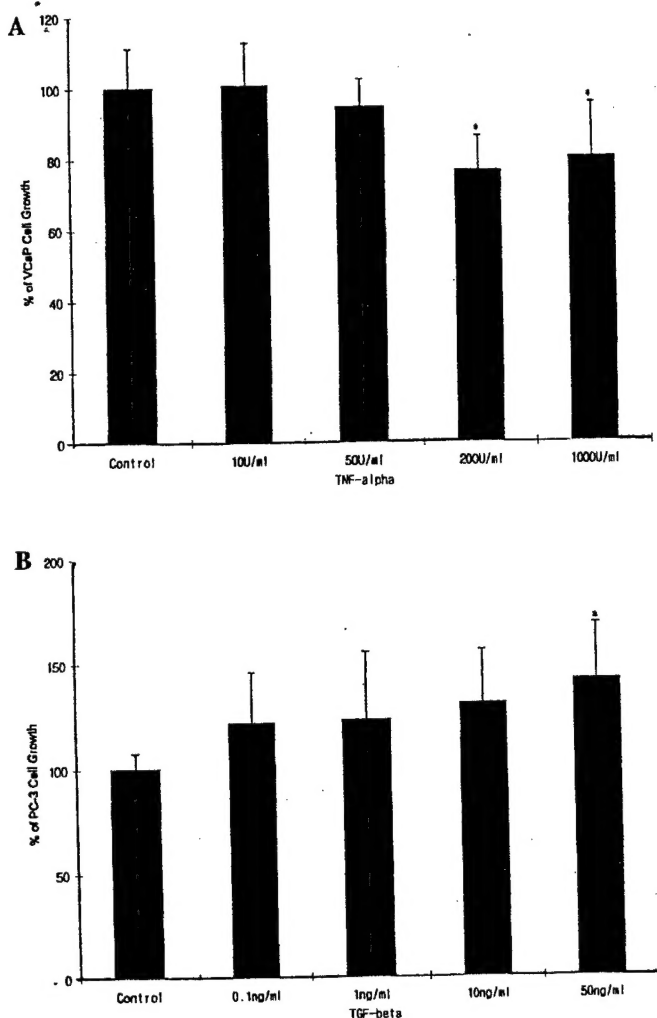


Figure 4. A, The growth response of VCaP cells incubated for 48 h in the presence of 0, 10, 50, 200, 1000 U/ml of TNF- α . B, The growth response of PC-3 cells incubated for 48 h in the presence of 0, 0.1, 1, 10, 50 ng/ml of TGF- β . Results (mean \pm SDM) expressed as the percent of the control, with an asterisk indicating significant values ($p < 0.05$) as compared to the control.

proliferation of MDA-2b cells, but it inhibited the proliferation of MDA-2a cells. PC-3 cell growth was also stimulated significantly ($p < 0.05$) by PDGF (Fig. 3A).

Although PDGF demonstrated a global, yet variable, effect on all cell lines derived from bone metastases, other GFs and cytokines also demonstrated an effect. IL-1 significantly stimulated the growth of MDA-2a and MDA-2b cells (Fig. 3B). VCaP and MDA-2a cells responded to bFGF, but the effects were variable (Fig. 3C). The growth of VCaP cells was significantly inhibited by bFGF at 2.5 ng/ml and 10 ng/ml, while all doses of bFGF stimulated the growth of MDA-2a cells ($p < 0.05$). VCaP cell growth was also reduced by approximately 20% when treated with more than 200 U/ml of TNF- α ($p < 0.05$) (Fig. 4A). Only the higher dose of TGF- β , 50 ng/ml, significantly stimulated the growth rate of PC-3 cells ($p < 0.05$) (Fig. 4B). The treatments of the four prostate cancer cell lines derived from bone metastases with IGF-1, EGF, and IL-6 did not demonstrate a significant effect (data not shown).

Discussion

IGF-1 is an important growth factor for mesenchymal tissues, including bone and cartilage, and plays an important role in the proliferation of human PCa cell lines (11-13). PC-3, LNCaP, and DU145 cells secrete IGF-1 and have IGF-1 receptors. These cell lines grow by an autocrine loop in which the overproduced IGF-1 activates its receptor (13). Ritchie and colleagues (14), suggested that GFs, such as the IGFs, can increase prostate cell metastasis by stimulating cell division at metastatic sites. The data regarding the role of exogenous IGFs on human PCa cell growth are conflicting. Exogenous IGFs have been shown to stimulate and suppress the proliferation of some PCa cell lines (14-18). The growth rate of LNCaP cells was reportedly decreased by exogenous IGF-1 (13). In this study, exogenous IGF-1 had a stimulatory effect on LNCaP cell growth.

Exogenous PDGF either inhibits or enhances the proliferation of prostate cancer cell lines *in vitro* (13,16, 19,20). In a previous study, exogenous PDGF stimulated the proliferation of PC-3 cells in a dose-dependent manner and overcame dexamethasone's inhibition of PC-3 cell growth (19). The treatment of LNCaP cells with PDGF did not stimulate their growth (20). In our study, PCa cells derived from soft tissue metastases were insensitive to PDGF, whereas PCa cells derived from bone metastases were sensitive to PDGF; however, the sensitivity of these cells to exogenous PDGF was variable, and the expression of PDGF receptors and the exact role of PDGF in PCa cell lines, were not determined.

It has been reported that the androgen-independent PC-3 and DU145 cells produce and are inhibited by TGF- β , while androgen-responsive LNCaP cells neither produce nor respond to TGF- β (22). These observations were confirmed in other investigations (14,19-21,23). In the present study, LNCaP cells did not respond to TGF- β , and this observation is in agreement with earlier publications. In contrast, the growth of PC-3 and DU145 cells was not affected by TGF- β . Surprisingly, the highest concentration of TGF- β , which was not used in earlier studies, stimulated the growth of PC-3 cells. Although a reason for the different outcomes is being determined, we speculate that both PC-3 and DU145 may have acquired an inactivating mutation in the gene encoding TGF- β type I receptor (24), similar to that detected in LNCaP cells that render them insensitive to the effect of TGF- β . Recently, it was reported that PC-3 and DU145 cells are genetically more unstable than LNCaP cells; therefore, it is conceivable that the PC-3 and DU145 cell lines in our laboratory may have different TGF- β type I receptor genotypes than those used for previous investigations (24,25).

EGF has been shown to be important in the development of several neoplasms (26). Several human prostate cancer cell lines, including PC-3, LNCaP, and DU145, express EGF and EGF-receptors. Published investigations suggest that autocrine activation of an EGF-receptor by EGF, regulates PCa cell growth (27,28). MacDonald and Habib (27) concluded that exogenous EGF only minimally affected the growth and DNA synthesis of DU145 cells, whereas LNCaP cell growth was stimulated. In contrast, this study demonstrated that LNCaP cells were insensitive to exogenous EGF, while

DU145 cell proliferation was significantly increased. Interestingly, all the bone-derived PCa cell lines failed to respond to exogenous EGF treatment. The expression of EGF receptors in our cell lines was not determined.

bFGF has been implicated in the osteoblastic response associated with PCa and other tumor cell lines (29,30). Nakamoto and colleagues (31) reported that DU145 and PC-3 cells produced active bFGF and expressed large amounts of bFGF receptors, though only DU145 responded to exogenous bFGF. Other studies observed that PC-3 and DU145 cells were insensitive to exogenous bFGF (16,21) and our data support these observations (16,21). The response of LNCaPs to bFGF is variable, with some studies reporting that bFGF stimulates LNCaP growth (32,33). The current study demonstrated that soft tissue cell lines did not respond to any dose of exogenous bFGF ranging from 0.1 to 10 ng/ml, but among cells derived from bony metastases, there were variable responses to exogenous bFGF. bFGF stimulated MDA-2a cell growth and inhibited VCaP cell growth. Other cells derived from bone metastases were insensitive to exogenous bFGF.

IL-1 receptors are expressed on PCa cell lines and, once, activated by IL-1, can alter their growth (34). Hsieh and Chiao (34) determined that IL-1-induced a dose-dependent growth reduction in LNCaP and JCA-1 cells. Abdul and Hoosein (35) reported that IL-1 α mRNA transcripts were expressed in PC-3 and DU145 cell lines but not in LNCaP, MDA-2a, and MDA-2b cell lines. IL-1 β transcripts were expressed only in the PC-3 cell line (35). They also demonstrated that LNCaP cell growth was significantly reduced by low concentrations of IL-1 α and β , but PC-3 and DU145 cells were not affected. Ritchie and colleagues (14) reported that TNF- α , and IL-1 β significantly inhibited the proliferation of LNCaP, DU145, and PC-3 cells. In the current study, only MDA-2a and 2b cells responded to exogenous IL-1 β by demonstrating an increase in their cell growth.

A variety of malignant tumors have been shown to express IL-6, which may use an autocrine loop as a possible mechanism for stimulating cancer cell growth (36-38). LNCaP, DU145, and PC-3 cells have receptors for IL-6, and this cytokine has been reported to inhibit LNCaP cell growth (39,40). Okamoto and colleagues (41) identified possible paracrine and autocrine pathways mediating the IL-6 effect in the LNCaP cell line. They reported that LNCaP cell growth was stimulated by exogenous IL-6 and also suggested that IL-6 functions as a paracrine growth factor for the LNCaP cell line and as an autocrine growth factor for DU145 and PC-3 cell lines. Chung and colleagues (42) reported that IL-6 inhibited the *in vitro* growth of LNCaP cells, but demonstrated no effect on the growth of PC-3 and DU145 cells. They also concluded that IL-6 appears to undergo a functional transition from paracrine growth inhibitor to autocrine growth stimulator during progression of PCa to the hormone-refractory phenotype. Other studies reported that exogenous IL-6 inhibited growth of LNCaP, PC-3, and DU145 cells (14,43). In our study, the growth of seven PCa cell lines was not modulated by exogenous IL-6.

TNF is a well-characterized secretory product of macrophages that has shown antitumor activity and is being investigated as an important agent in host-mediated antitumor defense (44,45). Sherwood and colleagues (46) demonstrated

that recombinant TNF- α (range 50-200 ng/ml) was cytotoxic to PC-3, DU145, and LNCaP cell lines but not to benign prostatic, epithelial and stromal cell lines *in vitro*. Other investigations demonstrated that TNF- α was cytotoxic to LNCaP cells but was not cytotoxic to PC-3 and DU145 cells (47-50). Out of seven tested PCa cell lines, only the growth of androgen-sensitive LNCaP and VCaP cells was inhibited by exogenous TNF- α in our study. Recently, several other researchers reported that nuclear factor- κ B (NF- κ B) had a protective role on TNF- α -induced apoptosis (51,52). Sumitomo and colleagues (47) demonstrated that only TNF- α -induced a slight inhibition in the growth of advanced PCa cell lines (PC-3 and DU145); however, inhibition of NF- κ B activity enhanced the ability of TNF- α to induce growth inhibition and apoptotic cell death. Based on our data, we speculate that PCa cell lines, taken from various metastatic sites, are relatively resistant to TNF- α treatment (50).

These results demonstrate that the responses of these cell lines to bone-associated GF and cytokines are variable and depend on the unique phenotype of each cell line and not their metastatic origin nor their expression of the androgen receptor. Prostate cancer metastasis to bone may not be mediated by preferential proliferation in the bone marrow in response to bone-associated GFs and cytokines.

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